

# Genomic Island 2 Is an Unstable Genetic Element Contributing to *Brucella* Lipopolysaccharide Spontaneous Smooth-to-Rough Dissociation<sup>∇†</sup>

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*Brucella* is a Gram-negative bacterium that causes a worldwide-distributed zoonosis. The genus includes smooth (S) and rough (R) species that differ in the presence or absence, respectively, of the O-polysaccharide of lipopolysaccharide. In *S. brucellae*, the O-polysaccharide is a critical diagnostic antigen and a virulence determinant. However, *S. brucellae* spontaneously dissociate into R forms, a problem in antigen and S vaccine production. Spontaneous R mutants of *Brucella abortus*, *Brucella melitensis*, and *Brucella suis* carried the chromosomal scar corresponding to genomic island 2 (GI-2) excision, an event causing the loss of the *wboA* and *wboB* O-polysaccharide genes, and the predicted excised circular intermediate was identified in *B. abortus*, *B. melitensis*, and *B. suis* cultures. Moreover, disruption of a putative phage integrase gene in *B. abortus* GI-2 caused a reduction in O-polysaccharide loss rates under conditions promoting S-R dissociation. However, spontaneous R mutants not carrying the GI-2 scar were also detected. These results demonstrate that the phage integrase-related GI-2 excision is a cause of S-R *brucella* dissociation and that other undescribed mechanisms must also be involved. In the R *Brucella* species, previous works have shown that *Brucella ovis* but not *Brucella canis* lacks GI-2, and a chromosomal scar identical to those in R mutants was observed. These results suggest that the phage integrase-promoted GI-2 excision played a role in *B. ovis* speciation and are consistent with other evidence, suggesting that this species and *B. canis* have emerged as two independent lineages.

Brucellosis is one of the most important bacterial zoonotic diseases. This infection is caused by the members of the genus *Brucella*, a group of Gram-negative, intracellular facultative microorganisms (13). *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella neotomae*, *Brucella ceti*, and *Brucella pinipedialis* are denominated smooth (S) *brucellae* because their colonies have glossy, bluish surfaces that contrast with the rough (R), granular aspect of the *Brucella canis* and *Brucella ovis* colonies. Moreover, S *brucellae* had a marked tendency to yield mixtures of S and R colonies, a phenomenon known as *Brucella* S-R dissociation (4). These colony phenotypes respectively relate to the presence or absence of the O-polysaccharide of lipopolysaccharide (LPS) (1, 14), and since this structure is a major diagnostic antigen and virulence factor, S-R dissociation has drawn considerable attention (1, 4, 14, 21). Indeed, S-R dissociation is a problem in the production of diagnostic antigens and vaccines (3). Although first observed over 70 years ago, the genetic bases of *Brucella* S-R dissociation have not been elucidated as of yet.

Up to now, 10 *Brucella* O-polysaccharide genes (putatively coding for enzymes furnishing sugar precursors, glycosyltransferases, and the O-chain transport system) have been identi-

fied, and the corresponding mutants show the R phenotype (1, 9). These genes are located in two major genetic regions, *wbo* and *wbk*. The *wboA* and *wboB* genes (encoding O-polysaccharide glycosyltransferases) are part of a genomic island (GI) known as GI-2 (17, 18). This island contains a putative phage integrase gene that could promote recombination between flanking repeat sequences, thus causing a deletion that would include those two glycosyltransferases. Accordingly, GI-2 could be unstable, and this could account at least in part for the tendency of S *brucellae* to dissociate into R variants. Here, we report the characterization of spontaneous R *Brucella* mutants carrying the complete deletion of GI-2 and the detection of the corresponding excised circular intermediate. We also provide experimental evidence for the role of the GI-2 phage integrase gene in promoting S-R dissociation.

## MATERIALS AND METHODS

**Bacterial strains, growth conditions, phenotype identification, and plasmids.** The strains used in this study are listed in Table 1. To prepare the inoculum for dissociation experiments, bacteria were grown in *Brucella* agar plates (Becton Dickinson, Sparks, MD) for 48 h at 37°C, and a loopful was transferred to a 50-ml Erlenmeyer flask with 10 ml of *Brucella* broth (Becton Dickinson). The broth was incubated with shaking until bacteria reached stationary phase (48 h at 37°C), an aliquot was adjusted at an  $A_{750}$  of  $0.109 \pm 0.005$  using sterile broth, and 100  $\mu$ l was used to inoculate a new 50-ml Erlenmeyer flask with 10 ml of broth supplemented with McIlvaine's buffer (pH 6.6) (4). This broth was incubated statically for 10 days at 37°C, bacterial colony counts were obtained by plating serial 10-fold dilutions, and S-R dissociation was assessed by using the crystal violet dye exclusion test (27). For each strain, two plates with approximately 1,500 colonies per plate (the limit for obtaining isolated colonies in 36 to 48 h; longer times produced confluent growth) were examined, and three independent experiments were performed. For cloning, *Escherichia coli* was grown in Luria-Bertani broth

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† Supplemental material for this article may be found at <http://jb.asm.org/>.

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TABLE 1. Bacterial strains

Strain	Characteristics	Source or reference <sup>a</sup>
<i>B. melitensis</i> 16M	<i>B. melitensis</i> biovar 1 reference strain; S	SAG
<i>B. suis</i> 1330	<i>B. suis</i> biovar 1 reference strain; S	SAG
<i>B. abortus</i> 2308	<i>B. abortus</i> biovar 1 reference strain; S	SAG
<i>B. abortus</i> 2308 Nal <sup>r</sup>	Nalidixic acid-resistant <i>B. abortus</i> 2308; S	UNAV
<i>B. ovis</i> 63/290	Reference strain; R	SAG
<i>B. abortus</i> B10	<i>B. abortus</i> biovar 1, Chilean field isolate; S	11
<i>B. abortus</i> R1	Spontaneous $\Delta$ GI-2 mutant derived from strain 2308; R	This study
<i>B. abortus</i> R6	Spontaneous $\Delta$ GI-2 mutant derived from strain B10; R	This study
<i>B. abortus int::Km<sup>r</sup></i>	<i>int</i> mutant (BAB1_0983), 2308 Nal <sup>r</sup> derivative; S	This study
<i>B. abortus int::Km<sup>r</sup>/int</i>	<i>int::Km<sup>r</sup></i> mutant complemented with plasmid pMR10int (carrying a copy of <i>int</i> ); S	This study
<i>E. coli</i> TOP10F	F <sup>-</sup> <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74</i> <i>recA1</i> <i>ara</i> $\Delta$ 139 $\Delta$ ( <i>ara-leu</i> )7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str <sup>r</sup> ) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>E. coli</i> OmniMAX 2-t1	F <sup>'</sup> [ <i>proAB<sup>+</sup> lacIq lacZ</i> $\Delta$ M15 Tn10(TetR) $\Delta$ ( <i>ccdAB</i> )] <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80( <i>lacZ</i> ) $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> ) <i>U169</i> <i>endA1</i> <i>recA1</i> <i>supE44</i> <i>thi-1</i> <i>gyrA96</i> <i>relA1</i> <i>tonA</i> <i>panD</i>	Invitrogen
<i>E. coli</i> S17-1 $\lambda$ pir	Mating strain with plasmid RP4 inserted into the chromosome	20

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(Becton Dickinson) supplemented with kanamycin (50  $\mu$ g/ml) or chloramphenicol (20  $\mu$ g/ml). The plasmids used in this work are listed in Table S1 in the supplemental material.

**Genetic analysis of spontaneous R mutants.** Identification of GI-2 scars in spontaneous R mutants was carried out first by PCR analysis (see below) of individual crystal violet-positive colonies (approximately 80 R colonies for *B. abortus* 2308 of a total of 3,000 colonies). The frequency of GI-2 deletion was then calculated with respect to the total number of R colonies examined. In addition, the numbers of GI-2 scars in the inocula used for plating were examined by quantitative PCR (qPCR) (see below). In this case, the frequency of detection of the GI-2 deletion was calculated with respect to the number of copies of the genome.

**Sequence and restriction analyses.** The complete sequences of *B. abortus* 2308 and *B. ovis* ATCC 25840 chromosome I were downloaded from GenBank (accession numbers AM040264 and CP000708, respectively). Similarity searches were performed by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). *In silico* restriction analysis of GI-2 was performed with NEBcutter v2.0 (<http://tools.neb.com/NEBcutter2/index.php>). Primers (see Table S2 in the supplemental material) were generated with Primer3 (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>). DNA sequencing by the dideoxy method was performed at Macrogen Inc., Seoul, South Korea.

**DNA extraction and PCR assays.** Genomic DNA (gDNA) was isolated from liquid cultures using standard protocols (28). Long-range PCR assays were performed in a final volume of 25  $\mu$ l containing 20 to 50 ng of gDNA, 0.4 mM deoxynucleoside triphosphate (dNTP), 12.5 pmol each of the BAB1\_0997F and BAB1\_1008R primers (see Table S2 in the supplemental material), and 1 U of long-range enzyme mix (Fermentas Inc., Burlington, Canada). The PCR conditions used included an initial step at 94°C for 2 min, followed by 30 cycles at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 68°C for 13 min. Finally, an extension step at 68°C for 10 min was performed. PCR products were identified by restriction analysis with PvuII (Fermentas). For chromosomal scar detection, PCR assays were performed in a final volume of 25  $\mu$ l containing 0.2 to 0.5  $\mu$ g of gDNA, 10 pmol each of BAB1\_0981F and BAB1\_1007R, 0.2 mM dNTPs, 2 mM MgCl<sub>2</sub>, and 1 U of *Taq* Platinum enzyme (Invitrogen, Carlsbad, CA). The mixture was preincubated at 95°C for 5 min, followed by 30 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The same conditions were used for circular intermediate (CI) detection using primer pair BAB1\_1005F and BAB1\_0983bR. All PCR were carried out in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Amplicons and restriction fragments were resolved by electrophoresis in 1.0 to 2.0% TAE (40 mM Tris-acetate, 1 mM EDTA; pH 8.0) agarose gels.

**qPCR.** The frequency of detection of GI-2 deletion was assessed by absolute quantification using real-time PCR with gDNA of the  $\Delta$ GI-2 strain (R1 strain) or plasmid DNA from the cloned 586-bp fragment carrying the *attB* site (see pGI2del in Table S1 in the supplemental material) to prepare calibration curves. Amplification rounds included 0.2  $\mu$ g of gDNA, 0.3  $\mu$ M each of the BAB1\_0981F and BAB1\_1007R primers (see Table S2 in the supplemental material), 4 mM MgCl<sub>2</sub>, 1  $\mu$ l of LightCycler FastStart DNA SYBR green I master mix (Roche, Indianapolis, IN), and PCR-grade water to a final volume of

20  $\mu$ l in a LightCycler1.5 real-time PCR system (Roche). The reaction was initiated by enzyme activation and DNA denaturation at 95°C for 10 min and 45 cycles at 95°C for 8 s, annealing at 65°C for 8 s, and extension at 72°C for 20 s. The specificity of the reaction was assessed by melting curve analysis using the LightCycler3 software. For this, the amplicons were heated to 95°C for 1 s and 70°C for 15 s and then heated slowly at 0.1°C/s to 99°C under continuous fluorescence monitoring. Each round included a negative control without DNA. The frequency of detection of GI-2 deletion was estimated from the ratio between the numbers of chromosomal scars and genome copies of the corresponding DNA sample assessed by *A*<sub>260</sub> (16). All measurements were done in duplicate. Three independent experiments were performed with each strain.

**Mutagenesis.** The construction of the *B. abortus int* mutant was accomplished by gene disruption (23). Briefly, primers BAB1\_0983-mutF and -R (see Table S2 in the supplemental material) were used to amplify an internal fragment of *int* using *B. abortus* 2308 Nal<sup>r</sup> DNA as the template. This fragment was inserted into the BamHI-XbaI-digested pJQK plasmid, and the resulting pJQKint plasmid was transferred to the *E. coli* S17-1 $\lambda$ pir strain and introduced into *Brucella* by mating. Integrative mutants were selected on nalidixic acid (25  $\mu$ g/ml) and kanamycin (50  $\mu$ g/ml). To confirm the site of the insertion, independent PCR rounds with primer pair BAB1\_0983\_F1 and M13R and primer pair BAB1\_0983\_R4 and M13R were performed (see Fig. S1 in the supplemental material). For complementation, a copy of *int* generated by PCR with primers BAB1\_0983F-gw and BAB1\_0983R-gw (see Table S2 in the supplemental material) was introduced in the plasmid vector pDONR221 using BP Clonase (Gateway technology; Invitrogen). The resulting plasmid pDONRint was used along with the pMR10 destination vector to obtain the complementation plasmid pMR10int by LR Clonase reaction and cloned into the *E. coli* OmniMAX 2-t1 strain. After this, the construct was introduced into the *B. abortus int::Km<sup>r</sup>* mutant using the *E. coli* S17-1 $\lambda$ pir procedure.

**RNA extraction and reverse transcription assays.** Total RNA was prepared from 10 ml of *B. abortus*, *B. melitensis*, or *B. suis* cultures using RNeasy (Qiagen, Hilden, Germany). DNA contamination was removed by treatment with Turbo DNase (Ambion, Austin, Texas), and the yield was measured at *A*<sub>260</sub>. For cDNA synthesis, 100 ng of total RNA was reverse transcribed using 10 units of Transcriptor RT (Roche) and 0.3  $\mu$ M BAB1\_0983F-specific primer. The reaction mixture was incubated at 55°C for 30 min, as described by the manufacturer (Roche). A 401-bp internal fragment of *int* was amplified in a reaction mixture containing 2  $\mu$ l of cDNA, 12.5 pmol of BAB1\_0983F and -R primers, 0.4 mM dNTPs, 2 mM MgCl<sub>2</sub>, 1 $\times$  PCR buffer, and 1 U of *Taq* Platinum (Invitrogen) with a final volume of 25  $\mu$ l. Samples were incubated at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. At the end, a final extension step at 72°C for 5 min was included. Reverse transcription-PCR (RT-PCR) was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems).

**IS711 Southern blotting.** The IS711 sequence was identified by Southern blotting using 1 to 2  $\mu$ g of AvaI-ClaI double-digested *B. abortus* gDNA. The product was resolved in 1% agarose at 15 mA for 10 h and probed with a biotin-labeled (BioNick; Invitrogen) IS711 fragment generated by PCR with primers 711u and 711d (15). Chemiluminescent detection of the hybridized

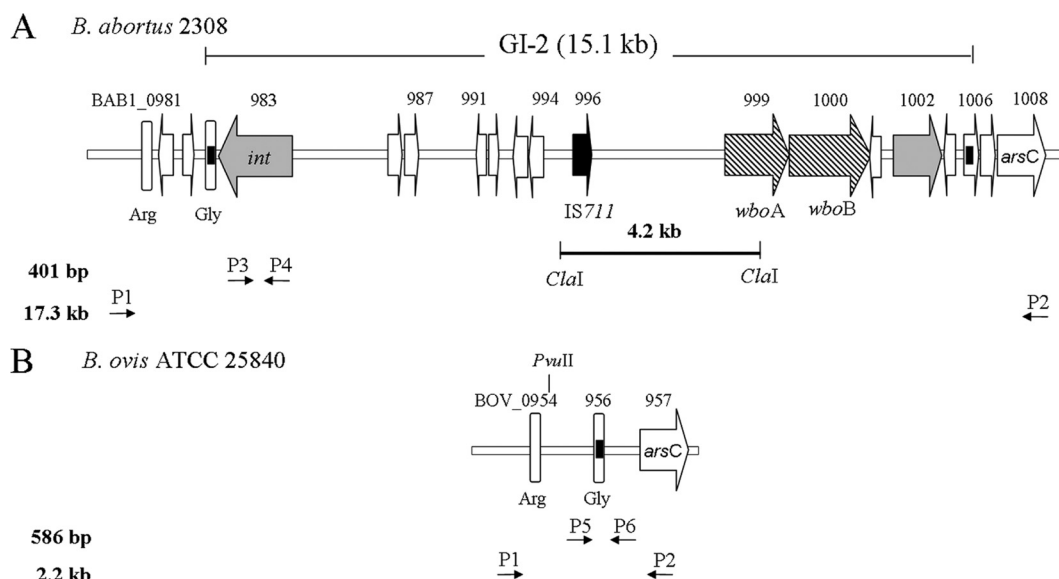


FIG. 1. Structure of *B. abortus* GI-2 and the corresponding *B. ovis* locus. (A) Genetic organization of *B. abortus* 2308 15.1-kb GI-2. Gray arrows, phage-related genes (*int*, integrase gene); cross-hatched arrows, LPS biosynthesis genes; white arrows, unknown or hypothetical ORFs; black arrow, *IS711* (containing the transposase ORF); white vertical bars, tRNA genes; black boxes (within the tRNA gene or a hypothetical ORF flanking the GI), direct repeats sequences. The positions of primers BAB1\_0979F and BAB1\_1008R (used to amplify a 17.3-kb fragment, including GI-2) are marked P1 and P2, and the positions of primers BAB1\_0983F and -R (used to amplify a 401-bp fragment of the integrase) are marked P3 and P4, respectively. The *ClaI*-*ClaI* segment indicates the position of the corresponding *IS711*-carrying 4.2-kb restriction fragment. (B) Genetic organization of the *B. ovis* ATCC 25840 locus carrying the GI-2 excision scar. In this case, primers P1 and P2 amplify a 2.2-kb fragment spanning the *attB*-containing scar (black box) and a *PvuII* site. The positions of primers BAB1\_0981F and BAB1\_1007R (used to amplify a 586-bp internal fragment of the scar) are marked P5 and P6, respectively.

product was done using a commercial kit (KPL, Gaithersburg, MD). Films were developed by conventional photographic methods.

**Nucleotide sequence accession numbers.** Sequences of PCR products derived from the scar and the CI were deposited to GenBank under accession numbers EU128673 and EU128674, respectively.

## RESULTS AND DISCUSSION

In *B. abortus* 2308, GI-2 is located in a 15.1-kb chromosomal region found between coordinates 953807 and 968958 of chromosome I (GenBank accession number AM040264) (5). This region complies with the structure of a canonical GI, as it is inserted into the tRNA<sup>Gly</sup> gene sequence flanked by a pair of 41-bp identical direct repeats. GI-2 carries 13 open reading frames (ORFs), with an average of 51.3% of GC content. This is lower than the average of 57% for the whole genome, suggesting horizontal acquisition (7). A putative phage-related integrase (BAB1\_0983) is located at the 5' end of GI-2, and the two genes (*wboA* and *wboB*) encoding the O-polysaccharide glycosyltransferases are located at the 3' end (Fig. 1A). *B. abortus* GI-2 shows 11 ORFs annotated as pseudogenes in comparison with the *B. melitensis* orthologs, as follows: a 25-kDa immunogenic protein precursor (BAB1\_0989-990), a queuine tRNA ribosyltransferase (BAB1\_0995), a phage-related protein (BAB1\_0998), and the hypothetical proteins BAB1\_0984-985, BAB1\_0988, BAB1\_0997, BAB1\_0998, and BAB1\_1004-1005. *In silico* restriction analysis of GI-2 predicted a 4.2-kb *ClaI* fragment carrying a copy of *IS711* (Fig. 1A). In contrast to the S species, *B. ovis* lacks GI-2 (22, 25). Instead, there is a chromosomal scar with a putative *attB* site (Fig. 1B), suggesting that GI-2 excision may have occurred

spontaneously and that this could be a cause of S-R dissociation in S *Brucella* species.

To assess the stability of GI-2, we grew *B. abortus* 2308 (S) (Table 1) in *Brucella* broth under standard conditions (37°C with continuous stirring) and performed long-range PCR for the detection of the complete GI-2 with primers BAB1\_0979F and BAB1\_1008R. Two amplicons were generated, one of approximately 17.3 kb that corresponded to the predicted product and a second one of 2.2 kb, which was similar to that amplified from *B. ovis* (Fig. 2A). These results suggest that a fraction of the cells in the *B. abortus* 2308 culture were undergoing the loss of GI-2. Then, we performed a similar experiment using *B. abortus* field isolate B10. Again, the same two types of PCR products were obtained (not shown). Moreover, restriction analysis of the *B. abortus* B10 2.2-kb PCR product showed a pattern similar to that obtained from *B. ovis* (Fig. 2B). We then selected two spontaneous R *B. abortus* mutants of different genetic backgrounds (R1 and R6) (Table 1) for further characterization. Like *B. ovis*, both mutants showed the *attB* site at the chromosomal scar and the absence of the GI-2 integrase (Fig. 2C). Sequencing of the product confirmed the identity of the scar, and the loss of GI-2 in the R1 and R6 chromosomes was further confirmed by the absence of a 4.2-kb *IS711*-containing fragment (Fig. 2D). Finally, we examined *B. abortus*, *B. melitensis*, and *B. suis* cultures for the presence of the circular intermediates (CIs) that should be released upon GI-2 excision (Fig. 3A). Consistent with this hypothesis, PCR targeted to the *attP* sites in the CIs using primers BAB1\_1005F and BAB1\_0983bR confirmed the release of GI-2 in *B. abortus*, *B. melitensis*, and *B. suis* (Fig. 3B). Moreover, sequence anal-

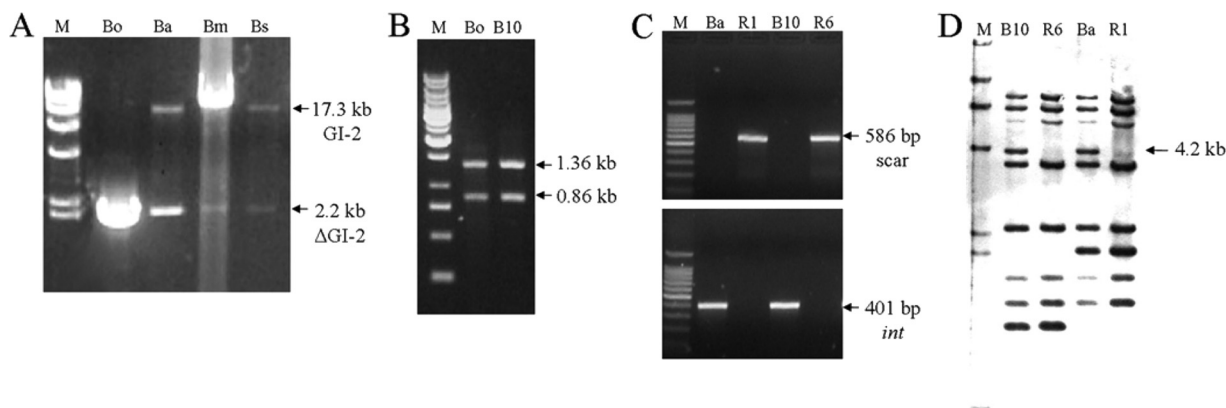


FIG. 2. Excision of GI-2 occurs spontaneously. (A) Long-range PCR analysis (primers P1 and P2) (Fig. 1) for the presence of GI-2 in *Brucella* cultures. Bo, *B. ovis*; Ba, *B. abortus* 2308; Bm, *B. melitensis* 16M; Bs, *B. suis* 1330. Abbreviations: 17.3-kb GI-2, GI-2-containing fragment (Fig. 1); 2.2-kb ΔGI-2, chromosomal scar carrying fragment (Fig. 1); M,  $\lambda$ /HindIII DNA ladder (Fermentas). (B) PvuII restriction analysis of the 2.2-kb fragment of the following: B10, *B. abortus* B10, and Bo, *B. ovis*. M, 1-kb DNA ladder (Fermentas). (C) PCR analysis of R. *abortus* mutants R1 and R6. (Top) Detection of the chromosomal scar with primers P5 and P6 (Fig. 1); (bottom) detection of the *int* gene with primers P3 and P4 (Fig. 1). M, 100-bp DNA ladder (Promega, Madison, WI). (D) IS711 Southern blot of the following: B10, *B. abortus* B10 (field isolate); R6, R. *abortus* mutant derived from B10; Ba, *B. abortus* 2308; and R1, R. *abortus* mutant derived from strain 2308. The arrow shows the position of the 4.2-kb ClaI restriction fragment (Fig. 1) that carries the IS711 copy. M,  $\lambda$ /HindIII biotin-labeled DNA ladder. All experiments were performed with DNA from broth cultures, except for 2C, in which DNA from a single bacterial colony was used.

ysis showed the 41-bp repeat at the *attP* and flanking *att* sites, indicating the occurrence of site-specific recombination (Fig. 3C). All together, these results link the spontaneous excision of GI-2 to the appearance of the R phenotype in these three *S. Brucella* species. We also examined the permanence of CIs in 5 spontaneous R mutants that carried the GI-2 deletion. None of these mutants were positive for CI detection by PCR, show-

ing that the CIs had been lost beyond detection, consistent with the absence of a replication origin.

It is known that low oxygen tension and acidic pH promote S-R *Brucella* dissociation and that R mutants tend to accumulate as cultures reach stationary phase (2, 4). By qPCR using the BAB1\_0981F and BAB1\_1007R primers, we estimated a GI deletion frequency of  $1.0 \pm 0.21 \times 10^{-5}$  to  $2.9 \pm 0.32 \times$

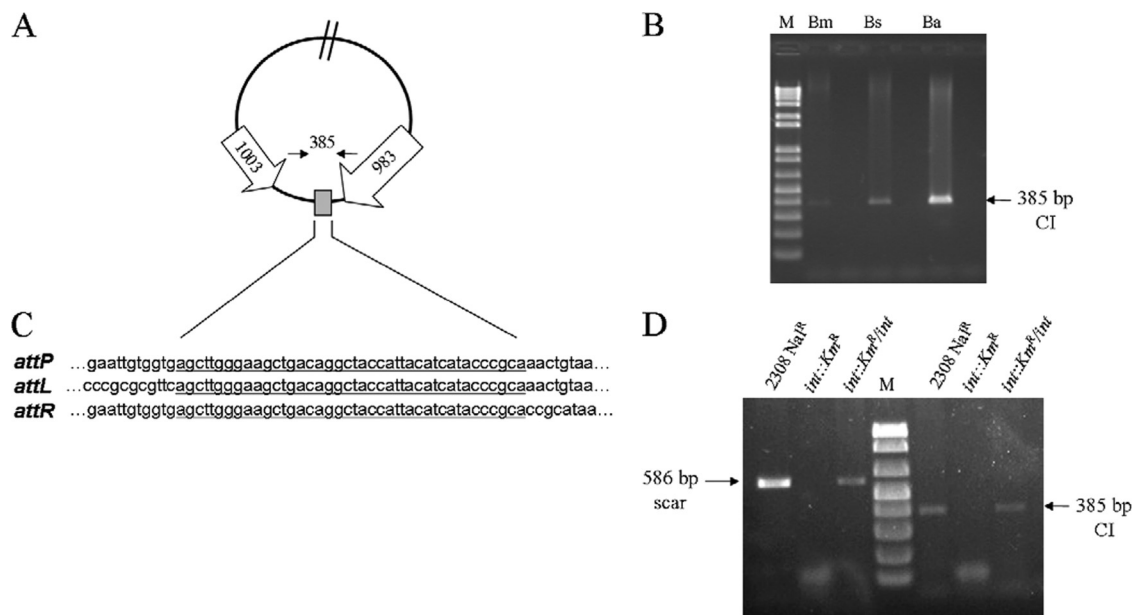


FIG. 3. GI-2 excision is promoted by the phage-related integrase gene. (A) Diagram of the excision intermediate showing the position of the 385-bp fragment carrying the recombined GI-2 repeats (gray box), amplified by PCR with primers BAB1\_1005F and BAB1\_0983bR. (B) PCR detection of the 385-bp product amplified from the excision intermediate in weak acidic cultures of *B. melitensis*, *B. suis*, and *B. abortus*. M, 1-kb ladder (Roche). (C) Sequence alignment of the flanking *att* and *attP* sites. The 41-bp repeat is underlined. (D) Mutational analysis of the phage-related integrase. DNA from the *B. abortus* parental (2308 NaI<sup>r</sup>), mutant (*int::Km<sup>r</sup>*), and complemented (*int::Km<sup>r</sup>/int*) strains was used for PCR detection of the GI-2 scar (586 bp) and CI (385 bp).



TABLE 2. Frequencies of S-R dissociation and of detection of the GI-2 deletion

Strain	Growth conditions	Dissociation frequency ( $10^{-2}$ ) <sup>a</sup>	Frequency of detection of GI-2 deletion by:	
			PCR ( $10^{-3}$ ) <sup>b</sup>	qPCR ( $10^{-5}$ ) <sup>c</sup>
<i>B. melitensis</i> 16M	37°C, pH 6.6, 10 days	0.1 ± 0.03	Not detected	10 ± 3
<i>B. suis</i> 1330	37°C, pH 6.6, 10 days	0.7 ± 0.10	Not detected	67 ± 15
<i>B. abortus</i> 2308	37°C, pH 6.6, 10 days	2.7 ± 0.25	4.5 ± 1.1	900 ± 210
<i>B. melitensis</i> 16M	37°C, late-stationary phase	Not detected	Not detected	1.0 ± 0.21
<i>B. suis</i> 1330	37°C, late-stationary phase	Not detected	Not detected	1.3 ± 0.25
<i>B. abortus</i> 2308	37°C, late-stationary phase	Not detected	Not detected	2.9 ± 0.32

<sup>a</sup> R colonies were identified by using the crystal violet dye exclusion test, and the dissociation frequency was calculated with respect to the total colony count.

<sup>b</sup> PCR detection of the chromosomal scar was carried out for each R colony, and then the GI-2 deletion frequency was assessed (values represent the means and standard errors of the means of results from three independent experiments).

<sup>c</sup> Values represent the means and standard errors of the means of results from three independent experiments. The frequency of detection of the GI-2 deletion was estimated as the ratio between the numbers of scars and genome copies. The efficiency (*E*) of the PCR obtained within the range of  $10^6$  to  $10^2$  target copies was  $1.84 \pm 0.07$ .

$10^{-5}$  for stationary-phase cells (Table 2). When we examined the dissociation of *B. abortus* 2308 using *Brucella* broth adjusted to pH 6.6 and prolonged the incubation for 10 days, we isolated R mutants with a frequency of  $2.7 \pm 0.25 \times 10^{-2}$  (Table 2), with the GI-2 deletion accounting for only 10 to 20% of them. Moreover, although qPCR evidenced the occurrence of the GI-2 deletion, we did not detect the GI-2 chromosomal scar in any of the *B. melitensis* and *B. suis* R mutants isolated on plates. These results suggest that in addition to GI-2 excision, there are other mechanisms involved in S-R *Brucella* dissociation. One of these mechanisms could involve the transposition of insertion element (IS) sequences into GI-2, as suggested by the fact that the spontaneous R mutant RB51 presents IS711 inserted in *wboA* (24). Our results also leave open the possibility that such alternative mechanisms of S-R dissociation could have different relevance in different *Brucella* strains or species. This is suggested by the different estimated frequencies observed by qPCR in *B. melitensis* 16M, *B. suis* 1330, and *B. abortus* 2308 grown under acidic conditions (Table 2). Research is in progress to study these possibilities.

We registered the expression of the putative integrase/recombinase (BAB1\_0983) gene in all cases tested (data not shown), and we hypothesized that it could be involved in the excision. Accordingly, we constructed a *B. abortus* 2308 *Nal<sup>r</sup> int::Km<sup>r</sup>* mutant by gene disruption (see Fig. S1 in the supplemental material) and the corresponding *int::Km<sup>r</sup>/int*-complemented derivative. By PCR, we identified both the chromosomal scar and the CIs from cultures of the parental strain and in the *int::Km<sup>r</sup>/int*-complemented strain but not in the mutant (Fig. 3D). In addition, the assessment of the S-R dissociation rates of *B. abortus* 2308 *Nal<sup>r</sup>* and *B. abortus* 2308 *int::Km<sup>r</sup>* demonstrated a stabilization of the S phenotype associated to the *int* mutation (Fig. 4).

Finally, it is worth commenting on the role of the S-R dissociation in the biology of the genus *Brucella*. It has been proposed that GI circularization facilitates its transfer within a bacterial population, thus enhancing the genetic adaptability and competitiveness during host-pathogen interactions (19). Also, it has been suggested that GI deletion is accompanied by the appearance of a less virulent phenotype, a result that could help some bacteria to succeed in long-term colonization during infection (10). However, these hypotheses are unlikely to be

relevant in *Brucella*. First, this pathogen shows high clonality, with no evidence of any mechanism of gene exchange at the population level, in keeping with its intracellular lifestyle (6). Second, it has been a consistent observation that spontaneous and laboratory-produced R *Brucella* mutants are severely attenuated in cells and animals (1, 21), making it unlikely that GI-2 deletion plays a role in the perpetuation of the disease. However, this does not mean that the excision does not happen within the host. In fact, the GI-2 chromosomal scar has been identified in DNA obtained from abomasum fluids of *B. abortus*-infected cattle (M. Mancilla, unpublished results), and one possibility is that the acidic environment of the abomasum might have facilitated GI-2 excision. The significance of the S-R dissociation in this and possibly other extracellular fluids is unclear. R *Brucella* mutants are also occasionally isolated from the milk of infected goats (C.M. Marín, J. M. Blasco, and I. Moriyón, unpublished observations), and they may represent variants that, although able to multiply in environments less stringent than the intracellular niche, are not transmitted from host to host. GI-2 instability may have played a role in speciation within the genus *Brucella*. Even though *B. ovis* and *B. canis* carry the major O-polysaccharide *wbk* genetic region, both are

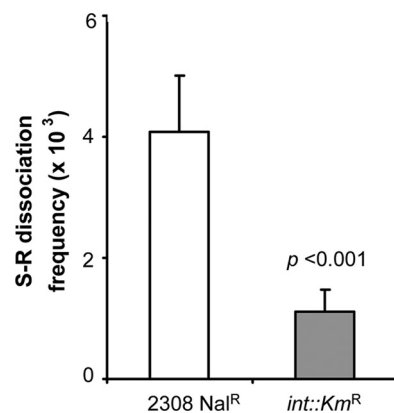


FIG. 4. Dissociation frequencies of the parental strain of *B. abortus* (2308 *Nal<sup>r</sup>*) and the mutant in the phage-related integrase gene (*int::Km<sup>r</sup>*). The means and standard errors of the means of the results from three independent experiments were  $4.1 \pm 0.9 \times 10^{-3}$  and  $1.1 \pm 0.3 \times 10^{-3}$ , respectively ( $P < 0.001$ ; chi-square test).

naturally rough (R) (8). Previous works have shown that *B. ovis* carries a frameshift in *wbkF* (29) and a GI-2 deletion (25), and here we provide evidence for the mechanism behind this deletion. On the other hand, *B. canis* conserves GI-2, and its R phenotype has been traced to a deletion overlapping *wbkD* and *wbkF* (29). These data show that the emergence of *B. canis* and *B. ovis* has followed different paths, consistent with the clustering of the former with *B. suis*, and the evidence suggests that *B. ovis* forms a more ancient lineage (12, 26).

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#### REFERENCES

- Allen, C. A., L. G. Adams, and T. A. Ficht. 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. *Infect. Immun.* **66**:1008–1016.
- Altenbern, R., D. Williams, J. Kelsh, and W. Mauzy. 1956. Metabolism and population changes in *Brucella abortus* II: terminal oxidation and oxygen tension in population changes. *J. Bacteriol.* **73**:697–702.
- Alton, G., L. Jones, R. Angus, and J. M. Verger (ed.). 1988. The production of *Brucella* vaccines. Techniques for the brucellosis laboratory. INRA, Paris, France.
- Braun, W. 1946. Dissociation in *Brucella abortus*: a demonstration of the role of inherent and environmental factors in bacterial variation. *J. Bacteriol.* **51**:327–349.
- Chain, P. S., D. J. Comerchi, M. E. Tolmasky, F. W. Larimer, S. A. Malfatti, L. M. Vergez, F. Aguero, M. L. Land, R. A. Ugalde, and E. Garcia. 2005. Whole-genome analyses of speciation events in pathogenic *Brucellae*. *Infect. Immun.* **73**:8353–8361.
- Ficht, T. A. 2003. Intracellular survival of *Brucella*: defining the link with persistence. *Vet. Microbiol.* **92**:213–223.
- Gal-Mor, O., and B. B. Finlay. 2006. Pathogenicity islands: a molecular toolbox for bacterial virulence. *Cell. Microbiol.* **8**:1707–1719.
- Godfroid, F., A. Cloeckaert, B. Taminiau, I. Danese, A. Tibor, X. de Bolle, P. Mertens, and J. J. Letesson. 2000. Genetic organisation of the lipopolysaccharide O-antigen biosynthesis region of *Brucella melitensis* 16M (*wbk*). *Res. Microbiol.* **151**:655–668.
- Gonzalez, D., M. J. Grillo, M. J. De Miguel, T. Ali, V. Arce-Gorvel, R. M. Delrue, R. Conde-Alvarez, P. Munoz, I. Lopez-Goni, M. Iriarte, C. M. Marin, A. Weintraub, G. Widmalm, M. Zygmunt, J. J. Letesson, J. P. Gorvel, J. M. Blasco, and I. Moriyon. 2008. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. *PLoS One* **3**:e2760.
- Hacker, J., U. Hentschel, and U. Dobrindt. 2003. Prokaryotic chromosomes and disease. *Science* **301**:790–793.
- Mancilla, M., M. Villarroel, M. E. Saldías, J. Soto, and A. M. Zárraga. 2008. Genotipos de aislados de campo de *Brucella abortus* de distintas regiones geográficas de Chile. *Arch. Med. Vet.* **40**:187–192.
- Moreno, E., A. Cloeckaert, and I. Moriyon. 2002. *Brucella* evolution and taxonomy. *Vet. Microbiol.* **90**:209–227.
- Moreno, E., and I. Moriyon. 2007. The genus *Brucella*, p. 315–455. In S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), *The prokaryotes*, 3rd ed., vol. 5. Springer-Verlag, New York, NY.
- Moriyon, I., M. J. Grillo, D. Monreal, D. Gonzalez, C. Marin, I. Lopez-Goni, R. C. Mainar-Jaime, E. Moreno, and J. M. Blasco. 2004. Rough vaccines in animal brucellosis: structural and genetic basis and present status. *Vet. Res.* **35**:1–38.
- Ocampo-Sosa, A. A., J. Aguero-Balbin, and J. M. Garcia-Lobo. 2005. Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 and the new subgroup 3b of biovar 3. *Vet. Microbiol.* **110**:41–51.
- Pavlovic, G., V. Burrus, B. Gintz, B. Decaris, and G. Guedon. 2004. Evolution of genomic islands by deletion and tandem accretion by site-specific recombination: ICES1-related elements from *Streptococcus thermophilus*. *Microbiology* **150**:759–774.
- Rajashekara, G., J. Covert, E. Peterson, L. Eskra, and G. Splitter. 2008. Genomic island 2 of *Brucella melitensis* is a major virulence determinant: functional analyses of genomic islands. *J. Bacteriol.* **190**:6243–6252.
- Rajashekara, G., J. D. Glasner, D. A. Glover, and G. A. Splitter. 2004. Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. *J. Bacteriol.* **186**:5040–5051.
- Schmidt, H., and M. Hensel. 2004. Pathogenicity islands in bacterial pathogenesis. *Clin. Microbiol. Rev.* **17**:14–56.
- Simon, R., U. Priefer, and A. Pehle. 1983. A broad host range mobilization system for in vitro genetic engineering: transposon mutagenesis in gram negative bacteria. *Biotechnology* **1**:784–890.
- Spink, W. 1956. The nature of brucellosis, p. 53–58. The University of Minnesota Press, Minneapolis, MN.
- Tsolis, R. M., R. Seshadri, R. L. Santos, F. J. Sangari, J. M. Lobo, M. F. de Jong, Q. Ren, G. Myers, L. M. Brinkac, W. C. Nelson, R. T. Deboy, S. Angiuoli, H. Khouri, G. Dimitrov, J. R. Robinson, S. Mulligan, R. L. Walker, P. E. Elzer, K. A. Hassan, and I. T. Paulsen. 2009. Genome degradation in *Brucella ovis* corresponds with narrowing of its host range and tissue tropism. *PLoS One* **4**:e5519.
- Uzureau, S., M. Godefroid, C. Deschamps, J. Lemaire, X. De Bolle, and J. J. Letesson. 2007. Mutations of the quorum sensing-dependent regulator VjbR lead to drastic surface modifications in *Brucella melitensis*. *J. Bacteriol.* **189**:6035–6047.
- Vemulapalli, R., J. R. McQuiston, G. G. Schurig, N. Sriranganathan, S. M. Halling, and S. M. Boyle. 1999. Identification of an IS711 element interrupting the *wboA* gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. *Clin. Diagn. Lab. Immunol.* **6**:760–764.
- Vizcaino, N., P. Caro-Hernandez, A. Cloeckaert, and L. Fernandez-Lago. 2004. DNA polymorphism in the *omp25/omp31* family of *Brucella* spp.: identification of a 1.7-kb inversion in *Brucella cetaceae* and of a 15.1-kb genomic island, absent from *Brucella ovis*, related to the synthesis of smooth lipopolysaccharide. *Microbes Infect.* **6**:821–834.
- Whatmore, A. M. 2009. Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. *Infect. Genet. Evol.* **9**:1168–1184.
- White, P. G., and J. B. Wilson. 1951. Differentiation of smooth and non-smooth colonies of brucellae. *J. Bacteriol.* **61**:239–240.
- Wilson, K. 2001. Preparation of genomic DNA from bacteria. *Curr. Protoc. Mol. Biol.* Chapter 2:Unit 2.4.
- Zygmunt, M. S., J. M. Blasco, J. J. Letesson, A. Cloeckaert, and I. Moriyon. 2009. DNA polymorphism analysis of *Brucella* lipopolysaccharide genes reveals marked differences in O-polysaccharide biosynthetic genes between smooth and rough *Brucella* species and novel species-specific markers. *BMC Microbiol.* **9**:92.